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IN NERVE CELLS

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Introduction

Membranes and their derivatives play a key role in all
biological systems, constituting about 40% to 90% of the
total mass in different cell types. They represent a
major structural component of nervous tissues, ranging
from individual limiting membranes of the wide variety of
cells to multiply folded or invaginated systems which are
often disposed in "paracrystalline" arrays.[1,2,3] Attention
has focused on these specialized lamellar systems, including
mitochondria and photoreceptors, as prototypes of organized
membranes which carry out energy transduction function
with remarkable efficiency. The highly ordered structural
framework of these cell membranes provides relatively stable
"modular floor-space," thereby ensuring periodic arrangement
of specialized macromolecular enzyme complexes and related
assemblies.[4,5,6]

It is possible, in light of accumulating evidence, that
these membrane systems may operate equally well as inform-
ation transducers. In fact, the neuron, which as the unit
of the nervous system is invested with the primary function
of information transfer, possesses membrane-bound terminal
endings with specialized synaptic structures that function-
ally interlink specific cell groups.[7] The suggested
location of subcellular or molecular switch gears at such
synaptic junctions manifests their integral association

with the processes of information storage, transfer, and
readout. According to Schmitt [7] the primary role of
molecular recognition of coded information stored in macro-
molecules of the brain cells, is the formation of the
exceedingly complex neuronal network of the human brain.
Some of the most sophisticated behavioral patterns of the
instinctual type involve primarily molecular recognition
of coded information stored in DNA-RNA-protein macromolecular
systems. The underlying molecular specificity in the key
junctional regions of the nervous system must ultimately
be taken into account in any attempts to explain the singular
capacity for accurate storage of the near infinite number of
memories which span a lifetime.

The pioneering polarized light [8] and x-ray diffraction
investigations of the nerve myelin sheath [9] provided a
standard of reference for the study of cell membrane ultra-
structure in general. However, the task of directly visual-
izing individual cell membranes, whose thickness is in the
order of 100 Å to 200 Å, remained for the higher resolving
power of the electron microscope. [10,11] Ultrastructural
analysis is now progressing beyond the initial stage of
delineating the membrane's lipoprotein framework.

Based essentially on the paucimolecular theory of membrane
structure proposed by Danielli and Davson, [12] Robertson has
propounded a unit membrane hypothesis. [13] After demonstra-
ting that plasma membranes of all cells and the membranes of

their organelles show certain common structural features,
he proposes that biological membranes are comprised of a
phospholipid bilayer system, 40 to 60 Å thick, which
is coated on both sides with monolayers of macromolecular
material, presumed to be proteins.

This unit membrane concept of Robertson serves as a useful
working hypothesis which must now be extended and modified
according to the large body of evidence provided by corre-
lated electron microscopic and biochemical investigations
during the past few years. These studies have revealed
specific macromolecular functional repeating units and the
counterpart structural elements in mitochondria, [14,15,16,17,18] chloro-
plasts, [19,20] retinal rod outer segments, [21,22,23] and in a large
number of membranes from different cell types. [24,25,26,27]

Investigations of nerve membranes and related structures
were recently carried out by high resolution electron
microscopy, using improved preparation and instrumentation
techniques, which have already proved successful in the
analysis of virus fine structure and other biological systems.
Increasing emphasis has been placed on the study of the
detailed organization of the enzyme and multienzyme complexes
intimately associated with cell membranes as the molecular
componentry ultimately responsible for the highly specific
energy and information transduction functions.

Only salient aspects can be given here of·the available
evidence bearing on organization within the plane of the
layers, and the existence of macromolecular repeating units
in membranes. Particular attention will be paid to corre-
lated biochemical and electron microscopic studies of well
characterized multienzyme complexes which are representative
of the types of macromolecular assemblies that may be found
in association with nerve membranes. .An attempt will be
made to show how this association of specific macromolecular
assemblies with the hydrated lipoprotein framework of the
nerve membranes may serve to fulfill many structural and
functional requirements which are unique to the nervous
system. Representative examples will illustrate the
organization of the nerve myelin sheath and junctional
regions of the nervous system. The role of water as an
integral structural component will also be discussed in
relation to the functional specificity of these key sites
of the nervous system.

Organization of Myelin Membranes

Elucidation of the general submicroscopic organization of
the nerve myelin sheath may be regarded as the corner stone
of membrane ultrastructure research. Nearly two decades ago,
F. O. Schmitt and his associates[9,28] gave the first detailed
account of the highly ordered, layered structure of the myelin
sheath, as deduced from their classic small-angle, x-ray[9]
diffraction studies of living nerve and earlier polarized

light analysis.[8,28]   The postulated regular, concentric
arrangement of the myelin sheath layers was subsequently
verified by direct observation in the electron microscope.[10,11,14,30]
Geren and Schmitt[29,31]   then showed that the myelin sheath
derives from invaginated Schwann cell membranes multiply
wrapped around the axon of embryonic peripheral nerve fibers.
In the morphogenetic process of myelination, leading to the
formation of this condensed, "liquid crystalline" membrane
system, the constituent lipid and protein components in the
limiting membranes of both axons and Schwann cells (or glia
in the central nervous system) play specific roles.

The x-ray diffraction pattern of fresh intact nerve, which
serves as a standard of reference for the study of myelin
structure, features a number of very well-defined, low-angle
reflections. These reflections exhibit a characteristic
alternation of intensities in the even and odd orders, and
can be accounted for as the first five orders of a fundamental
spacing, varying from about 170 Å in amphibians to 178 Å
(Fig. 1A) to 184 Å in mammalian peripheral nerve.[9,32,33] The
low-angle x-ray diffraction patterns recorded from normal
nerve furnish general information on the dimensions and
approximate distribution of scattering groups in the radial
direction of the myelin sheath. Earlier studies of physical
and chemical modifications in the myelin sheath established
a correspondence between the layer spacings observed in the
electron micrographs (Fig. 1A) and the fundamental radial

repeating unit indicated by the low-angle x-ray diffraction patterns (Fig. 1C).

It should be pointed out, however, that the concentric laminated structure observed in electron micrographs of thin nerve sections is simply a pattern of the selective deposition of osmium or other reagents at certain sites, and it cannot be interpreted in terms of specific regions containing lipids, lipoproteins, or protein constituents of the myelin sheath. Moreover, extensive modifications are introduced by complex fixation, dehydration and embedding artifacts which must be taken into account in a critical evaluation of the fine structures observed in thin sections.[33,34] With these reservations in mind, it is assumed that the framework of the fundamental radial unit of the sheath is formed by two adjacent bimolecular layers of mixed lipids about 60 Å in thickness; each bilayer is coated with sheets of hydrated protein or other polysaccharide, non-lipid macromolecular material. The two bimolecular layers are distinguished by a "difference factor" which can be accounted for by assuming that in the process of myelin formation, the asymmetric wrapping of the Schwann cell membrane around the axon will produce a symmetry difference in successive layers.

Lipid Composition of Myelin

Relatively little is known about the composition of the major lipids and their fatty acids in myelin, since this component is very difficult to isolate reliably and in sufficient

quantity. Recently, however, J. S. O'Brien and associates [35] have reported the results of their efforts to determine the composition of lipids and fatty acids obtained from carefully isolated myelin, gray matter, and white matter in normal human frontal lobes at various ages. The lipid content of myelin, ranging between 78% and 81% of the dry weight, was found to be much higher than the lipid content of gray or white matter. According to the pioneering studies of Folch-Pi [36] and other earlier workers, myelin contains much higher molar proportions of cerebroside sulfate and plasmalogenes and slightly higher molar proportions of cholesterol than gray matter. It is noteworthy that in myelin the content of sphingolipids, containing very long-chain fatty acids (19 to 26 carbon atoms), is proportionately ten times that of any other membrane structure analyzed. Also, 1 in 17 fatty acids is polyunsaturated in the myelin group of lipids, while in the gray matter group the corresponding value is 1 in 5.

The recent molecular model of myelin proposed by Vandenheuvel [37] serves to illustrate certain features characteristic of myelin lipids which could account for the highly stable membrane structure. Lipids in myelin are not covalently bound. The three major forces holding these molecules within

the membrane result from: 1) electrostatic interactions between polar groups of lipids and oppositely charged groups in adjacent proteins, 2) hydrogen bonding between oxygen and nitrogen atoms in lipids and adjacent proteins, and 3) London-van der Waals dispersion forces between CH_2 pairs in hydrocarbon tails of adjacent lipid molecules. The latter may be the major force holding such molecules together, since the total force owing to the additive interactions of the large number of CH_2 pairs is appreciable. On the basis of steric hindrance, it can be expected that lipids containing polyunsaturated acids will not be held as rigidly in a bimolecular leaflet, thus leading to a more free and less-stable structure. The higher proportion of saturated glycerophosphatides present in myelin will therefore impart greater stability, since the lipid molecules can be more closely packed than in the membrane structures of gray matter which have a significantly higher content of polyunsaturated glycerophosphatides. Finally, the sphingo-lipids containing long-chain fatty acids of the type found in myelin can form interdigitated cholesterol-sphingo-myelin complexes as proposed by Vandenheuvel. All of this new data makes it possible to account for the closely packed and highly ordered myelin membrane structure. It may also help explain the remarkable degree of metabolic inertness of myelin lipids   which has   been derived from turnover studies with labelled lipids. [38,39] Myelin is thus considered to

be the most stable membrane known, lasting the lifetime of the animal.

All of these conclusions relate mainly to the lipid composition, but our knowledge about the structure, composition, and function of other constituents of myelin, including proteins, water, polysaccharides and salts is still very meager. Recent experiments by M. Singer and M. Salpeter[40] indicate that tritium-labelled L-histidine is transported through the Schwann and myelin sheath into the axon of peripheral nerves. Further studies along these lines should help clarify how incorporated amino acids traverse the myelin sheath.

Schmitt[41] has emphasized that the unique properties of the axon surface membrane appear to be determined by the inner protein component which is considered to be different from the bimolecular lipid phase and the external non-lipid layer. The inner monolayer of globular protein molecules, forming a continuous, mosaic sheet, may be capable of responding to electrical and ionic changes with fast conformation changes of the protein molecules. Such dynamic changes of protein molecules in relation to the adjoining lipid bilayer may significantly alter the net fixed charge, and consequently, the membrane or action potential. Schmitt and co-workers have given the name "electrogenic protein" to this membrane-associated protein capable of rapid conformation change in response to electric field or other bioelectric

modifications. Identification and localization of the
electrogenic protein may eventually be possible by combining
the immuno-neurological experiments of Huneeus-Cox et al[42]
with appropriately labelled antibody techniques for electron
microscopy, possibly using cytochrome-C which is smaller
than ferritin. The typical combination of the antibody or
-SH reagent with the electrogenic protein may also make it
possible to introduce selective labelling with heavy metals
for high resolution electron microscopy.

Subunit Structure of Myelin Membranes

Earlier investigations[10,11,14,30,33] had shown that the
layers of the myelin sheath tend to dissociate into granular
or rod-shaped particles about 60 $\overset{o}{A}$. This transformation occurs
following a wide variety of controlled physical and chemical
treatments which include enzymatic digestion, freezing and
thawing of nerve, and low-temperature fixation and embedding
techniques (Fig. 1A). Correlated electron microscopic and x-ray
diffraction data also indicated the possibility of a regular
organization within the plane of the layers, probably involv-
ing units of 60 to 80 $\overset{o}{A}$.[33] In vitro nerve degeneration
studies[4,6] have disclosed various forms of granular dissoci-
ation in the lamellae which closely resemble structures
observed after enzymatic digestion. It was determined from
low-angle, x-ray diffraction patterns that modifications of
the x-ray scattering power within the radial unit corresponded
with the rate of degeneration and were likewise consistent
with the presence of a granular structure in the

dense and intermediate layers of the myelin sheath. These results and consistent electron microscopic findings indicate a regular fine structure within the concentric arrays of the dense and intermediate layers (Fig. 1A) which warrants careful consideration.

Our investigations made use of cryofixation techniques which are based on rapid freezing of fresh or glycerinated specimens with liquid helium II at 1 to 2° K, or with undercooled nitrogen.[4,6] This is followed by freeze-substitution and embedding in plastics at low temperatures. Conditions are maintained which minimize ice-crystal formation and artifacts of chemical fixation, extraction, and embedding. These and related techniques for thin sectioning have consistently yielded better morphological and histochemical preservation of all types of lamellar systems and membrane derivatives than the standard procedures.[4,21,34] As shown in Fig. 1A, particulate subunit structures of 60 to 80 Å are regularly found, mainly within the plane of the intermediate layers, but also in the dense layers of the well-preserved concentric laminated structure. This type of structure was seen informally in all kinds of peripheral and central optic nerves, particularly of frog and rats. It is detected in cross sections, longitudinal sections, and particularly in tangential sections revealing face views of the plane of the layers.

Special techniques were also applied in preparing ultrathin, frozen sections of fresh tissue. They were cut with a special

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microtome and diamond knife in a cryostat at -30 to -180° C.[6,11,15]
The specimens were often examined directly without thawing
by embedding in vitrified heavy metal layers. A liquid
nitrogen cooling device was used in conjunction with appro-
priate low-temperature electron microscopy techniques.
Ultrathin frozen sections of fresh frog sciatic nerve,
negatively stained with phosphotungstate (Fig. 2B), show
characteristic repeating particulate units (arrows) of
about 50 to 60 Å. These units, mainly localized in the
intermediate layers and attached to the dense myelin layers,
were consistently found in the negatively stained, unfixed
specimens. Bearing in mind inherent artifact possibilities
(e.g., myelin figures), these and related methods are now
being further investigated in an attempt to examine the fine
structure of the myelin sheath under conditions closely
approximating its native hydrated state.

Recent work by Branton[44] and Moor[45] using the freeze-
etching technique show that the fracture faces of completely
myelinated membranes generally appear smooth and relatively
free of particles, in contrast to the inner faces of all
other membranes which show distinct particulate components.
These findings are of considerable significance because no
heavy metal staining is involved. However, pretreatment of
nerves in 20% glycerol, followed by freezing, is seen in our
earlier low-temperature, x-ray diffraction studies[4,6] to
produce characteristic modifications in the hydrated lipo-

protein constituents, often with underlying drastic altera-
tions of the hydrated matrix permeating the entire sheath.
In view of the limited level of resolution and the degree
of possible artifacts inherent in freeze etching when
dealing with such fine structures, further work is clearly
indicated.

Organization of Membranes in Photoreceptors

Membranes of the vertebrate photoreceptors comprise "unit
discs" which are stacked in regular order in the outer
segments. Earlier polarized light studies[8] indicated that
the rod outer segments consist of thin, transversely arranged
protein layers which alternate with longitudinally oriented
layers of lipid molecules. Electron microscopy has confirmed
this idea by revealing the fine structure of the outer
segment and its composition of several hundred unit discs
about 150 to 200 Å thick.[2,4,30,49]

Improved low-temperature electron microscopy techniques
revealed an intermediate layer between the dense layers,
and electron-dense structures within the layers which
appear as globular subunits (Fig. 3A).[6,21] These subunits
look well organized in dark-adapted outer segments and may
be related to the presence of the photopigment complexes,
as suggested by data from polarized light analysis and
experimental modifications. Since rhodopsin represents
about 40% of the dry weight in frog retinal rod outer
segments, it may be regarded as a principal structural

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component of the photoreceptor membrane system.

Because of its inherent uncertainties, however, electron
microscopical data on the fine structure of membrane in vivo
cannot be considered reliable unless confirmed by an inde-
pendent technique such as x-ray diffraction analysis.
Previous studies of this type[50] demonstrated a spacing of
about 320 Å which was tentatively correlated with the
repeating units observed along the axis of the rod outer
segments in osmium-fixed preparations. Although these low-
angle, x-ray diffraction patterns revealed layering of the
membranes and the intermembrane spacings, they did not yield
any specific information on the structure within the plane of
the membranes. Recent low-angle, x-ray diffraction studies
by Blasie and Dewey, Blaurock and Worthington,[51] have sub-
stantiated the presence of an orderly, globular substructure
within the isolated outer segment membranes of photoreceptors
in frog retina.

The x-ray diffraction patterns gave reflections which are
consistent with a square array of particles within the
membranes with a unit cell size of 70 Å. Low-angle, x-ray
diffraction showed periodicities in isolated outer segments
to be identical with those of intact outer segments from
the retinal receptors. The authors also observed an
orderly arrangement of particles about 40 Å in diameter
within negatively stained preparations of outer segment

membranes. However, our limited knowledge of the chemical composition of photoreceptors precludes interpretation of these particulate subunits in terms of specific photopigments, or associated multienzyme components.

We have used special preparation techniques and low-temperature electron microscopy to examine outer segments of the frog retinal rod. Fresh, ultrathin sections of light and dark-adapted segments were prepared without fixing and without embedding. The specimens were kept frozen or dried and were stained, negatively or positively, with buffered phosphotungstate, uranyl formate, and other electron-dense reagents. Additional structural detail can be detected in these well-preserved preparations (Fig. 3B,C). The globular subunits (60 to 70 $\mathring{A}$), which are prominent in the dense and intermediate layers, appear to be built up of minute particles, about 10 to 15 $\mathring{A}$, regularly arranged in clusters or rows. Symmetrical alignment of these dense particles is usually maintained, even in certain regions where the dense layers are split.

These preparation techniques, which combine the advantages of thin sectioning with the enhanced preservation of negative staining applied to native, unextracted specimens, [6,15] are particularly suitable for correlated biochemical studies. Moreover, when used in combination with enzymological assays of isolated retinal outer segments [52] it should be possible

to obtain essential data on the localization of the visual
pigments, with associated lipoprotein components and specific
enzyme systems.

Invertebrate Photoreceptors

Invertebrate photoreceptors are essentially composed of
highly ordered tubular compartments which are considered
differentiated membrane extensions, or microvilli, of the
retinula cell membrane. Closely packed arrays of these
tubules (ca. 400 to 1,000 Å in diameter) comprise the
radially disposed rhabdomeres of the retinula cells in each
ommatidium of the insect compound eye.

Earlier electron microscope studies [53,54,55] and more recent
electrophysiological investigations by Waterman et al [56] point
to the complex arrangement of photopigments within the
plane of the membranes as a key to an integral understanding
of the photoreceptor system. For example, the insect eye's
remarkable capacity for analyzing polarized light depends
essentially on the dichroism of the photopigment molecules,
which must possess a highly ordered "paracrystalline"
arrangement within the microvilli in order to provide the
molecular basis for differential sensitivity to linearly
polarized light. The precise orientation of the many
thousands of closely packed tubular compartments in a single
rhabdomere comprise a unit dichroic analyzer at the cellular
and subcellular levels. Recent data by Waterman and Horch, [56]

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which includes a model for a two-channel polarization analyzer, indicates that one ommatidium suffices for detecting small e-vector orientations.

The differentiated membrane-bound components in these photo-receptors display a highly ordered and symmetrical arrangement throughout the various dimensional levels of organization. Ultimately, however, this exquisitely built, compact system owes its capability of extracting optimum information, through selective interaction with incoming light signals, to the precise three-dimensional arrangement of the photopigment molecules within the membranes.

Macromolecular Repeating Units of Structure and Function in Mitochondrial Membranes

A large body of evidence has accumulated during the past decade[4,6,15,16,17,18] showing that the electron transfer system, the respiratory transformations, and certain other systems of the mitochondrion are built up from characteristic macromolecular complexes. Large scale isolation of stable mitochondria under conditions which do not impair their main enzymatic activities, play a significant role by making these membranous organelles and their constituents available for biochemical analysis.

Correlated electron microscopic and biochemical studies[6,15]

have resulted in the detection of a macromolecular repeating
particle associated with the cristae and the inner membrane
of the external mitochondrial envelope. Many thousands
(ca. 10^4 to 10^5) of these particles, are found disposed in
regular arrays in a single mitochondrion using negative stain-
ing and other preparation techniques. The repeating particle,
designated the mitochondrial elementary particle (EP), consist
of three parts: a spherical or polyhedral head piece, 80 to
100 Å in diameter; a stalk (ca. 50 Å long); and a base piece
(40 x 110 Å). As shown in Figures 4 A,B the base pieces of
the elementary particles form an integral part of the outer
dense layers of the cristae. These characteristic repeating
particles are particularly well demonstrated in negatively
stained specimens of mitochondria in situ, of isolated
mitochondria, and of submitochondrial membrane fragments
with a complete electron transfer chain.

Following the first observations by Fernández-Morán[6,14,15]
numerous investigators[16,17,18,60,61] confirmed the existence
of the repeating particles and underlying subunit organization
of mitochondrial membranes using a wide variety of techniques,
including the improved freeze-etching method applied by Moor
and Muhlethaler.[62] Since this technique operates on native
frozen specimens and does not involve the use of chemical
fixatives or stains, the successful demonstration of globular
repeating units in mitochondria and other membrane systems
has effectively disposed of tentative suppositions[63] that
these characteristic units might be preparative artifacts.

It is, therefore, now generally agreed by most workers [64,65,66,61] that these mitochondrial subunits exist, although the observed structural details will depend on the preparation techniques and experimental conditions.

Detection of these structural subunits of mitochondrial membranes suggested the existence of counterpart macro-molecular functional units. A particulate unit containing a complete electron transfer chain was isolated from beef heart mitochondria.[15,59] The repeating unit has a molecular weight of about 1.3×10^6 (corresponding to approximately 40 molecules of protein and 400 molecules of phospholipids). The experimental evidence obtained from our studies was consistent with the notion that the isolated (Fig. 4C) and re-constituted particles could be identified with the elementary particles (EP) visualized in situ. It was also assumed that the isolated elementary particles contained the complete electron transfer chain.

Although subsequent studies by Green and Perdue [17] indicate that there may be multiple species of mitochondrial elementary particles, the basic concept of a macromolecular repeating unit of mitochondrial structure and function is now generally accepted. The elementary particle of the mitochondrion is believed to be a prototype of a class of functional particles or macromolecular assemblies found in association with membranes generally.

Macromolecular Repeating Units in Chloroplast Membranes and other Membranes

Investigations by Park and Pon and others[19,20] have led to the identification of repeating units in the chloroplast membranes which are called quantasomes and may be the morphological expression of the physiological photosynthetic unit. X-ray diffraction work by Kreutz also indicates that the chloroplast lamellae, are built up of repeating units about 71 Å in diameter. The molecular weight of a single quantasome is 2×10^6. The interesting relationship between the quantasome of chloroplasts and the elementary particle of mitochondria has been pointed out by Park, et al. [20] Both are associated with the electron-transport system in membranes and both contain certain cytochromes, substituted benzoquinones and non-heme iron. Morphological differences have been revealed, however, in that four or more subunits appear in the quantasome. Branton and Park[46] show a second type of subunit, 110 Å in average diameter, which forms part of a matrix around the larger 175 Å quantasome units. It is concluded that the chloroplast lamellae consist of a matrix within which are densely packed subunits that form the major constituents of the photosynthetic membrane.

Plasma membranes of liver cells,[26,27] microvilli,[25] microsomal membranes,[45] bacterial membranes,[17] and synaptic membranes[47] are amongthe different types in which repeating units have been observed. In general, there is evidence that such

macromolecular repeating units appear in all membranes and
either make up the entire membrane or comprise a major part
of the membrane framework in close association with the
bilayer lipid structure. Although these repeating units are
of variable shape and size, with a range of molecular weights
from 50,000 to several million, they all have significant
features in common with the macromolecular assemblies
conceived by F. O. Schmitt [48] as distinctive hierarchial
units of structure and function (Fig. 4D) invested with
unique properties.

It has been shown that enzymes constitute a large propor-
tion of the total protein, particularly of specialized
energy transducing mitochondrial membranes. This catalytic
protein, may therefore be regarded as the key determinant
of the structural and functional organization of membrane
systems. In all membrane systems, including nerve membranes,
specific enzymes or enzyme complexes are attached to, or
otherwise intimately associated with, the basic membrane
framework in repetitive patterns of exquisite, three-
dimensional, stereospecific configuration.

The concept of macromolecular repeating units or assemblies
in membranes can therefore, in many ways, be regarded as
an extension of the unit membrane hypothesis of Davson,
Danielli [12], and Robertson [13], which represents a necessary
and logical transition consistent with present biochemical
and physico-chemical evidence.

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Macromolecular Organization of Enzyme Systems

A. Pyruvate and α-Ketoglutarate Dehydrogenation Complexes

A unique opportunity to correlate functional properties with ultrastructure has recently been provided by biochemical and electron microscopic studies of the pyruvate dehydrogenase complex (PDC) of Escherichia coli.[70] This well-characterized multienzyme complex, with a molecular weight of about 4.8 million, has been isolated in highly purified form and analyzed by Lester Reed and associates.[67,68]

PDC catalyzes a multistage oxidative decarboxylation of pyruvate,[67,71] and is composed of three enzymes. Each molecule of the PDC complex contains about 16 molecules of pyruvate decarboxylase (M.W. 183,000), 8 molecules of dihydrolipoic dehydrogenase (M.W. 112,000), and about 64 molecules of lipoic reductase-transacetylase (LRT). Lester Reed and his associates have also succeeded in reconstituting the complex from the isolated enzymes.[68] The concept of the organization of the PDC complex which emerged from these classical biochemical studies is that of an organized assembly of enzymes in which each of the constituent enzymes is specifically located to permit efficient implementation of a consecutive reaction sequence.

Our correlative electron microscope studies[69,70] have confirmed and extended this picture through direct visualization of the exquisite molecular architecture of the PDC complex and its components. The negatively stained PDC

complex appears in electron micrographs (Figure 5A) as polyhedrical particles of about 300 to 400 Å in diameter featuring a central tetrad (Figure 5B).

Electron microscopy of the isolated LRT aggregate revealed a typical tetrad structure which closely resembles the central tetrad of the native PDC complex and appears to determine its overall structure. According to the model derived from the biochemical and electron-microscopic data, lipoic reductase transacetylase (LTR) is composed of 64 identical subunit s situated at the eight vertices of a cube, while the molecules of pyruvate decarboxylase and dihydrolipoic dehydrogenase are respectively aligned on the twelve edges and in the six faces of the cube.[70,71]

The macromolecular organization of the α-ketoglutarate dehydrogenase complex (KGDC) isolated from **E. coli** appears to be similar to that of the PDC.[71,72] As shown by electron microscopy (Fig. 5C,D), the smaller polyhedrical particles, with a diameter of about 280 Å, feature a tetrad core surrounded by peripheral subunits, which are not clearly defined as in the PDC complexes.   Recent studies by Reed and his associates[71] indicate that the macromolecular

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organization of the bacterial and mammalian PDC and KGDC
multienzyme complexes are fundamentally similar, and may be
governed by the principles of self-assembly which determine
the functional organization of protein shells of regular
viruses with icosahedral symmetry.[73]

B. Organization of L-Glutamate Dehydrogenase

L-Glutamate dehydrogenase (GDH), which has a molecular weight
of 1 million, consists of four major subunits. This molecule
is of interest since it is known to be reversibly dissociated
by certain steroids and hormones such as thyroxin.[74]   Dis-
aggregation into much smaller units of about 40,000 molecular
weight occurs at high and low pH values or on treatment with
various agents.

Earlier electron microscope studies by Hall[75] revealed
particles about 145 Å wide and 80 Å high which were obtained
from dilute L-glutamate dehydrogenase (GDH) solution.
Valentine[76] observed spherical particles of 125 Å diameter,
90 Å and 75 Å in negatively stained GDH preparations.
Although the objects varied in size, depending on the con-
centration of the protein, these electron micrographs were
consistent with the concept of the undissociated molecule as
a tetramer. Subsequent observations by Horne and Grevel[77]
indicate that the molecules tend to dissociate into typical
tetrahedral subunits. However, attempts to observe the
undissociated molecules have been hampered by technical
difficulties.

Our recent investigations of bovine L-glutamate dehydrogenase crystals[78], using micro-droplet cross-spraying techniques and micro-beam illumination for high resolution electron microscopy, have made it possible to observe the ordered aggregation and fine structure of the relatively well preserved molecules in the thin microcrystals. As shown in Figures 6 A,B,C,D, individual spherical or polyhedral particles of diameter 130 to 150 $\overset{o}{A}$ can be frequently observed, either free (Figure 6 C,D) or closely packed in ordered aggregates (Figure 6B) of the thin crystalline strands. The individual polyhedral particles of diameter 130 to 150 $\overset{o}{A}$ may correspond to the undissociated individual molecules, while the numerous subunits of triangular shape could correspond partly to the GDH tetrahedral subunits described by Horne and Grevel.[77] However, on the basis of available data, the organization of the undissociated molecules appears to be more complex than the suggested simple aggregate of four subunits. Further studies are being pursued in attempts to elucidate the detailed subunit structure of these enzyme molecules. Investigation of the postulated changes in enzyme activity which may be produced by alterations of the subunit conformation of t hese enzyme molecules, can also be of significance in relation to fundamental controlling mechanism in cells. Moreover, this enzyme is also of interest as a model system to illustrate the different stages of molecular and supramolecular organization in the observed paracrystalline arrays, which are relevant to the general problem of the organization of enzyme complexes in membranes.

C. Fraction-I Protein

We wish to refer to another example of the characterization
of the substructure of a protein enzyme, which can be achieved
by combined electron microscopic and biochemical studies.
Fraction-I protein, which is a major soluble protein fraction
located predominantly in the chloroplast and which comprises
at least 50% of the soluble proteins, has marked carboxy-
dismutase activity. Based on recent studies carried out in
collaboration with Haselkorn, Kieras, and Van Bruggen, [79]
Fraction-I protein of Chinese cabbage leaves is seen to consist
of uniform cubical particles (Figure 7A) with an edge of
about 120 Å.

In high resolution electron micrographs of negatively stained
preparations (Figure 7B) the cubical particles exhibit a
characteristic subunit structure which is consistent with
a model having 24 subunits, in agreement with the present
physical and chemical data. In the proposed model, the sub-
units may undergo considerable conformational changes, and
do not appear to be related by principle of quasi-equivalence
which governs the structural organization of many of the
regular viruses.   The fact that the substructure of a
protein enzyme of only 120 Å can now be directly observed
in electron micrographs, is of particular relevance to
investigation of the enzyme complexes of similar size,
which are presumably associated with nerve membranes and
derivatives.

Membrane Ultrastructure in Nerve Cells - H. Fernández-Morán

Subunit Patterns of Neuronal Membranes

A. Structural Subunit Patterns of Synaptic Membranes

Investigation of the molecular organization of the synapse
is of particular significance in view of the specialized
molecular switching componentry assumed to mediate information
storage, transfer and retrieval at these junctional regions.

Unfortunately, neuronal membranes have not yet yielded to
correlate biochemical and electron microscopic studies of
the type successfully applied to mitochondrial and chloro-
plast membranes. Difficulties in reliably isolating and
preparing these labile structures without introducing
serious artifacts impose serious limitations.

Typical subunit structural patterns in certain kinds of
synaptic membranes have now been revealed by Robertson.[47]
His comprehensive electron microscopic studies demonstrate
a subunit pattern in the synaptic membranes of club-endings
of Mauthner cells of the goldfish. Each of the alternating
synaptic discs shows an internal beading with a repeat
period of 90 $\overset{o}{A}$ to 95 $\overset{o}{A}$ in vertical sections. Transverse
densities or lines spaced at 90 $\overset{o}{A}$ are seen in oblique sections,
while frontal views of the membranes reveal a honeycomb
pattern of lines and dots, approximately a hexagonal array.
This network features dense granules in the center of each
subunit facet and fine rodlet borders less than 20 $\overset{o}{A}$ in
diameter. The resemblance of these patterns to the structure
of Buckminster Fuller's geodesic domes suggests that this

type of efficient design may generally be embodied in the organization of cell membranes and regular virus shells.[47,73]

Similar synaptic subunit structures have been seen in the Mueller cell of goldfish medulla.[47,80] Confirming and extending our earlier results,[6,21] Robertson has demonstrated the existence of a regular subunit structure in the lamellae of retinal rod outer segments and in erythrocyte membranes.[80] This subunit pattern, which can be more readily seen when two membranes are closely apposed, is therefore, regarded as a structural pattern of general significance.[6,15,80]

Robertson assumes as a working hypothesis that this pattern represents a structural protein on the outer surface of the membrane.[80] Certain transverse densities which give the impression of a transverse globular substructure of the membranes are regarded with reservations since they may prove to be artifacts.[80] However, only further work with improved preparation techniques and appropriate x-ray diffraction analysis can establish or preclude the existence of such a regular subunit organization in native undegraded neuronal membranes.

B. Organization of Synaptic Membrane Complexes

Several interesting ultrastructural features have been revealed in synaptic membrane complexes of different types.[80,81] Gray[81] describes in mammalian spinal cord ventral horn cells synaptic membrane complexes which exhibit 500 Å dense particles

in regular hexagonal array with a 1,000 Å period. Certain
preparation techniques reveal presynaptic fibrils and
delicate hook-like fibrillar extensions from the post-
synaptic cytoplasm, making contact in the synaptic cleft.
In pyramidal cell synapses a patched differentiation of the
synaptolemma is observed with two types of interlemmal
elements which repeat at periods of about 235 Å.[80] An
unusual subsynaptic formation has been described by Taxi[80,81]
in autonomic ganglia of certain amphibia. This complex is
characterized by subsynaptic dense bands and granular regions
forming a triangle with the subsynaptic membrane as its base.
Identification and isolation of the specific macromolecular
componentry subserving the complex processes at these
junctional regions appears to be one of the most worthwhile
goals requiring the full repertory of ultrastructural,
chemical, and physiological investigations.

C. Fine Structure of Isolated Neuronal Membranes
The finest nerve fibers and neuronal extensions are fortun-
ately of truly paucimolecular dimensions and, therefore,
ideally suited for direct examination by electron microscopy.
Confirming and extending our earlier work,[11,30,82] certain
submicroscopic nerve fibers were found in frog spinal cord
usually attached to the thicker nerve fibers. These fibers
are thin enough to display their entire structure in a
single electron micrograph. As shown in Fig. 8 A, these
fibers appear as thin ribbons with diameters ranging between
0.1μ and 1μ and reaching lengths up to 50μ to 100μ.

Essentially, each fiber as displayed in a whole-mount preparation, consists of a thin sheath formed by a single tubular membrane which contains long filaments 100 to 200 Å in diameter. In our original shadowed and osmium-fixed preparations no definite indications of fine, regular structure were observed in these membranes. However, with improved techniques new ultrastructural detail (Fig. 8 B) was revealed in these well-preserved, ultra-thin nerve membrane specimens. They exhibit a characteristic surface structure featuring in certain regions hexagonal arrays of closely packed polygonal elements 50 to 90 Å in diameter. Various type of dense granular components are found attached to the membranes, particularly in junctional regions as shown in Figs. 8 B and 9 A,B.

These techniques can undoubtedly be considerably refined, and it should eventually be possible to examine these ultrathin submicroscopic nerve fibers in vacuum-tight microchambers under conditions approaching the native hydrated state.

The Role of Water in Membranes

Water is a major constituent of nerve membranes, representing at least 35% to 40%, even in condensed multilayered, fluid-crystalline systems like myelin.[2] The water layers at the aqueous interfaces of the fundamental repeating unit in myelin are about 12 Å to 15 Å thick. Their thickness appears to be largely determined by the electrical charge

density at the interfaces, and by the ionic strength.[2,3]

Depending on the different environments we may envisage various types of water structures including water within the membrane structure itself, water in direct contact with the ordered macromolecular membrane surfaces, and water layers between closely paired systems of unit membranes.[91] Interconnected water channels within the myelin layers and related lamellar systems play an important role as possible pathways of diffusion of ions and certain molecules between the axon and extracellular fluids.[3]

On the basis of results derived from a broad interdisciplinary approach[91,98] we must begin to consider water as a structured matrix, "the matrix of life" as was first pointed out by Szent-Györgyi.[89] Substantial experimental evidence is now available in support of a mixture model to account for the unique properties of water.[90] Thus, from neutron scattering data[97] and other studies of water we are now able to envisage a sample of liquid water as being made up of "flickering clusters" of about 50 to 100 molecules with an average lifetime of the order of 10^{-11} seconds, which is long enough for a structure to have a distinguishable existence.

The concept of organized water as an integral structural component of biological membranes must be given serious consideration.[2,6,88] We must assume that ordered water will specifically become integrated in the highly organized three-

dimensional macromolecular structures of living systems.
The abundance of postulated bulky species of water offers
numerous possibilities, ranging from the crystalline
hydrate structures to other frameworks of approximately
tetrahedral fourfold coordination.

Based on our earlier correlated studies of water using
nondestructive methods, such as nuclear magnetic resonance[2,4,34]
and x-ray diffraction techniques,[4,14] the concept was developed
of a three-dimensional hydrated lipoprotein system which provides
a general structural framework for specialized macromolecular
repeating units periodically arranged in the membrane layers.[4,5,6,15]
Although the main functional differences between membranes
would depend upon the types of transducing units present and
the complementary stereospecific configuration of the under-
lying substrate, this hydrated lipoprotein matrix could intro-
duce a common unifying feature. The water, particularly in
close association with the ordered lipoprotein membrane
systems, must likewise be highly ordered, resembling "ice-like"
hydration sheaths or crystalline hydrate lattices.

The open structure of water favors the formation of these
crystalline hydrates[94] which are clathrate compounds with a
hydrogen-bonded, ice-like framework defining cavities able
to enclose molecules of noble gases, liquids, ions, protein
side chains, etc., of appropriate size (4 to 6.9 $\mathring{A}$). The
hydrogen-bonded lattice of these microcrystalline hydrates,
which are about 12 $\mathring{A}$ to 24 $\mathring{A}$, is stabilized by van der Waals

interaction, in contrast to ordinary ice. The hydrate crystals
may therefore remain stable even in the range of normal body
temperatures under specialized conditions. As suggested
earlier,[5,6,87,88] these polyhedral hydrate structures with
numerous built-in cavities could effectively enclose ions,
or form closely fitting "molecular replicas" around protein
side chains, thus adapting to the specific macromolecular
configurations by three-dimensional interpenetration.
Crystalline hydrate structures of this general type, or
equivalent organized water structures permeating the ordered
lipoprotein-subunit macromolecular assembly system would
provide an interconnected H-bonded substrate for fast protonic
charge-transport mechanisms.[100]

Localized reversible phase changes in such ordered water
structures could also provide the basis for a variety of
conformational changes in protein and lipoprotein layers,
including reversible transitions from coherent bimolecular
lipid layers to the subunit micellar arrays. Reversible
phase transitions of this kind, spreading through the membrane
matrix, may contribute significantly to the propagation of
local perturbations. Likewise, selective permeability may
be envisaged in terms of molecular "pores" lined with ordered
water. In general, this concept of ordered water lattices
forming an integral structural component of membranes provides
a powerful conceptual basis for understanding many fundamental
mechanisms of membrane function,[91,98] as pointed out by Hechter.

The precise molecular nature of the ordered water in membranes is, however, still unknown. Nevertheless, nuclear magnetic resonance techniques have been successully applied in studies on the hydration structure of fibrous macromolecules.[99] Despite the uncertainties involved, there is recent evidence supporting the structural role of water in membrane systems. For example, Person and Zipper[91] have shown that treatment of mitochondrial suspensions with a dry zeolite, which clathrates the water, produces the same type of membrane disruption as is usually achieved with detergents. This novel form of membrane disruption can, therefore, be attributed to the avidity of the zeolite preparation for water, and its capacity to selectively remove certain divalent cations.

Extending our earlier work,[88,92,93] we have tried to obtain direct experimental verification of organized water structures of the crystalline hydrate type through controlled local formation of microcrystalline, noble gas hydrates in selected lipid, lipoprotein complexes, and cell membranes.[98] As shown in Figure 10 B characteristic electron-dense microcrystalline hydrates, about 10 Å to 20 Å in diameter, are localized mainly in the hydrophilic regions of lipid micelles following application of argon and xenon under controlled high pressure and temperature in special specimen chambers. The noble gas atoms hereby occupy polyhedral cavities in a hydrogen-bonded framework of water molecules, contributing to stabilize and make electron optically visible any pre-existing organized water structures of the hydrate type in lipid or lipoprotein model systems.

These techniques for direct visualization of noble gas hydrates could eventually provide experimental data bearing on the existence of the hydrate microcrystals reversibly forming in cell membranes. This approach is relevant to the molecular theory of general anesthesia postulated by Pauling.[95] According to this theory the formation of clathrate-like structures by anesthetic agents, including noble gases, in the aqueous portion of nervous tissues could cause anesthesia by modifying the electrical activity at junctional regions through entrapment of ions and electrically charged side chains of protein molecules. The formation of these hydrates would increase the structured water in the membrane systems, thus presumably making the water at synapses less available for facilitating conduction.

One might even go beyond this theory of Pauling, and in common with the earlier hypotheses of anesthesia advanced by Claude Bernard,[96] postulate reversible phase changes or "coagulations" in the synaptic membranes. It might not be unreasonable to assume that these reversible transformations in nerve membranes are not only associated with anesthesia, but perhaps also with certain rhythmic changes such as sleep.

Reversible modifications of the molecular organization involving primarily the hydrated lipoprotein framework of nerve membranes must, therefore, ultimately take into account the structure and cooperative ordering of water.

MEMBRANE ULTRASTRUCTURE IN NERVE CELLS - H. Fernández-Morán

The Biosynthesis of Membranes

The general problem of membrane biosynthesis is of funda-
mental importance for all biological studies concerned
with embryogenesis, morphogenesis, and differentiation.
The available evidence indicates that characteristic mech-
anisms are involved in the biosynthesis of the protein and
lipid membrane components, and also suggest the concept that
membranes may furnish the template for their "self-duplica-
tion".[109,110]

Investigation of nerve membrane biosynthesis may prove to
be of particular value in elucidating the synthesis and
assembly of the repeating subunits and their organization
within the plane of the membrane layers. The nature of
the association of the protein synthetic machinery and of
nucleic acids with cell membranes is therefore being studied
by electron microscopy and biochemical techniques to serve
as a basis for a better understanding of membrane biosyn-
thesis.

A. **Mitochondrial DNA, RNA, and Protein Biosynthesis System**
in Membrane Replication.

The discovery of characteristic nucleic acids in mitochon-
dria[101,102,103] and demonstration of a protein biosynthesis
system have recently confirmed and extended earlier work

(102) to provide significant support for the semi-indepen-
dence or autonomy of these organelles. DNA of mitochondria
from various mammalian cell types[103,104] differ from nuclear
DNA in guanine plus cytosine content, and in their renatura-
tion properties. In contrast to nuclear DNA which is linear,
the molecules of mitochondrial DNA exist in circular form,
their mean contour lengths measuring 5.24μ-5.45μ. Two to
six ring molecules of molecular weight 10×10^{6} may be
present in a single mitochondrion. Attachment of membrane
pieces to DNA, and the observed association of mitochondrial
DNA with cristae may possibly represent sites where replica-
tion is initiated.[104] Recent evidence also indicates that
mitochondria contain mechanisms for incorporation of nucleo-
tides into this DNA.[121]

Accumulating evidence indicates that in addition to DNA, RNA,
ribosome-like particles[102], and RNA polymerase-like systems[66,102]
have been found in mitochondria from different sources.

This new information furnishes the basis for a working
hypothesis implying that mitochondria possess their own gen-
etic information and the necessary mechanism to express this
information in an autonomous synthesis of proteins.[66] A 5.5μ
long DNA molecule is able to code the information for the
synthesis of several hundred proteins. As pointed out by
André,[66] part of this information may function as structural

genes, controlling the duplication of mitochondria and the synthesis of the biochemical equipment. Another part could function as regulatory genes, controlling differentiation and response to the external medium. Further studies will help to establish to what extent these genes are hypothetical, and to what extent they are subordinate to information coming from the nucleus, or may be reacting independently to the external medium by mutation and adaptation.[66]

These hypothetical considerations may prove to be of operational significance in view of the characteristic association of mitochondria with synaptic endings.[80,81] In monkey spinal cord motor horn cells Bodian has described very large endings containing unusually densely packed mitochondria. The subsynaptic cytoplasm in many of these large endings is characterized by an accumulation of Nissl substance which consists of regular layers of endoplasmic reticulum cisternae with large numbers of ribosomes in between. It has been speculated that these Nissl bodies may be associated with protein synthesis in relation to specific endings.[80] However, in view of the dense accumulation of mitochondria possessing their own genetic information and the mechanisms to express it in an autonomous synthesis of protein, the interesting possibility of a more direct participation of mitochondria in regulatory aspects of these biosynthetic processes should be taken into consideration. Subunits of protein molecules would be synthesized locally and either be attached as specific

macromolecular assemblies corresponding to coding and inform-
ation storage in molecular switching componentry at the
junctional regions, or they might pass through the ER membrane
and the cysternal cavity into the extracellular space.[80]
Similar considerations might also apply to the intimate inter-
relationship of mitochondria and associated granular components
with photoreceptors of the insect retinula as shown in Fig. 11.

B.   Role of RNA Polymerase in transfer of Genetic Information.
The enzyme RNA polymerase plays a key role in the transfer of
genetic information through its participation in the differ-
ential RNA transcription upon DNA templates.[106,107] Confirming
the results of earlier workers[105] we have studied the physical
properties of DNA-dependent RNA polymerase known to be relatively
free of nucleic acid and capable of asymmetric transcription.

As shown in Fig. 12 b, the RNA polymerase molecules from E.
coli, prepared by the Chamberlin and Berg procedure, and
having a sedimentation coefficient of 25 s, appear to consist
of six subunits arranged in a hexagon with a cross section of
120 to 130 $\overset{\circ}{A}$ in negatively stained preparations. If the large
hexagonal structures are the RNA polymerase molecules, as
proposed by Fuchs, Zillig et al, the basis structure is either
a hexagonal disc or two of these stacked together. A few smaller
structures are also observed which might be degradation or
dissociation products, some of which closely resemble the sub-
units in the hexagon. As shown by J. P. Richardson,[107] the
ionic conditions strongly affect the form of the RNA polymerase.

The low salt conditions favorable for observing the 21 s form
are the optimal conditions for enzymatic activity, while the
high salt conditions which produce the 13 s form completely
inhibit RNA polymerase activity.

In general, these observations emphasize the critical effect
of ionic conditions on all important systems participating
in the process of biosynthesis. Bearing this in mind, one
is tempted to speculate on the possible effect of ionic
currents which might have been modulated by bioelectric
potentials at key junctional membrane regions, thus pre-
sumably affecting the processes which regulate information
transfer and storage in nerve membranes and their derivatives.

In order to obtain more information on the manner in which
RNA polymerase initiates transcription of RNA upon DNA
templates, we are carrying out correlated electron microscopic
studies on the binding of RNA polymerase to different types
of DNA. Following the work of H. S. Slayter and C. E. Hall,[106]
and J. P. Richardson[107] it is assumed that certain sites must
exist along the DNA molecules which specifically bind RNA
polymerase, allowing transcription to begin at well-defined
regions. Preparations of RNA polymerase were mixed in with
different types of DNA, including $\emptyset$ X 174 DNA, bacterial and
salmon DNA, in varying concentrations under carefully
controlled conditions.

As shown in a representative example (Fig. 12 a) the complexed

enzyme generally appears to center itself upon the DNA axis,
which is in agreement with the results reported by Slayter
and Hall. Distribution of the RNA polymerase molecules along
the DNA fibers varied according to the different preparative
conditions. However, the disposition of enzyme particles
is similar to the distribution reported by Slayter and Hall,
with average separations ranging from about 1,200 $\overset{o}{A}$ to
about 2,000 $\overset{o}{A}$. These preliminary results are in agreement
with values reported by other workers. However, further
studies with improved preparation techniques are required
before a reliable evaluation can be carried out. Particular
interest attaches to correlated studies on the binding of
RNA polymerase to circular forms of DNA (e.g. from $\emptyset$ X 174)
which are being carried out in collaboration with Dr. Samuel
Weiss and his associates.

C. Morphopoietic Genes and Membrane. Biosynthesis.
As indicated earlier, the physical principles underlying
the designs of regular virus shells[73] may be applicable
to cell membranes assuming that the constituent protein or
lipoprotein subunits are organized in a lattice structure.
When dealing with the simplest virus shells we can envisage
that the subunits have complete shape-specificity, and thus
contain all the information needed for self-assembly of
identical subunits required to build up the prescribed shape.

More complex shells require additional information which is
stored in morphopoietic genes.[122] In T4 bacteriophage the

functions of three such genes are known, which appear to require a structural scaffolding by means of which this information is expressed.

There is reason to believe that we will have to cope with a major problem in this area when trying to understand the organization and functioning of cell membranes and their derivatives, which represent the next higher order of molecular structure in cellular organization. Knowledge of the genetic control of shape in protein structures will probably not be enough to explain the origin of the complex membrane-bound shapes of the highly specialized cells in the nervous system. However, in view of the complex relationships involved in membrane morphogenesis and differentiation, high resolution electron microscopy may be expected to play an important role in direct visualization of the sequential molecular patterns involved in nerve membrane biosynthesis.

Membrane Ultrastructure in Nerve Cells - H. Fernández-Morán

Cooperative Features of Nerve Membranes.

Biological membranes are the largest and most complex
atomic aggregates known, spanning the widest range of
dimensions as basic integrating components of all living
systems. Elucidation of the molecular organization of cell
membranes represents, therefore, not only one of the major
fundamental problems of biomedical research, but it has
also emerged as the next major barrier, cutting across
molecular biology and neurobiology, which must be overcome
to clear the way for further progress.

Membranes exhibit a unique degree of structural asymmetry,
comprising a coherent lipoprotein film that is usually only
a few molecules thick, but still capable of extraordinary
lateral extension.

Nerve membranes exhibit these characteristics to a unique
degree, as major components of the nervous system, which
is generally regarded as the most sophisticated and complex
biological system known. Thus, the total length of the
peripheral network of the human nervous system is esti-
mated at about 600,000 miles. All fibers of the human
body's nerve fabric, which is essentially lined with multi-
ply folded membranes, would extend three times the distance
from the earth to the moon. Having unravelled their
exquisitely delicate, yet highly ordered texture, we cannot
help wondering about the nature of these "paucimolecular"
"living" films, singularly sensitive to "paucienergetic

stimuli", [108] which are so compactly infolded into the sub-
microscopic recesses of the relatively limited space occupied
by the individual nervous system, that we could encompass a
whole planet with the combined array of extended membranes.

In many respects, the entire complex membrane repository
comprising the nervous system may be regarded as representing
an essentially interconnected, continuous and "closed membrane
system" where all junctional domains of the separate cell
membrane entities establish functional contact and are inter-
related to form an integrated, coherent whole of extraordinary
dimensions. In this sense, if a diamond crystal may be
considered as one giant "macromolecule" so it is justified
to conceive of these CNS membrane aggregates as one giant
"megamembrane" or "megamolecule". Such a coherent system
would be uniquely suited to effect transfer, storage and
retrieval of information at the various hierarchies of
organization.

Although this model is strongly idealized, it would, never-
theless, embody the basic features to serve as a suitable
substrate for highly cooperative phenomena embracing the
entire complex molecular aggregates, which are not unlike
those involved with manifestation of quantum phenomena at
the macroscopic scale, like for example, in superconducti-
vity. Although admittedly speculative and only intended to
delineate salient features of a model, this approach may
nevertheless prove to be a convenient working hypothesis
which could provide the operational key to a deeper under-

standing of the molecular and supramolecular organization features of the CNS.

Macromolecular Repeating Units and Domains in Membranes.
There is another feature of nerve membranes, which although derived from the results of ultrastructure research, never-theless, attempts to correlate them with the overall behavior of the system. Thus, membranes may also be regarded as arrays of organized domains or subunits, including macro-molecular assemblies. Katchalsky, et al[123] conceive of such cooperative systems as exhibiting "memory-hysteresis behavior". It is pointed out that there exists also the possibility of a physical record in biopolymers based on conformational changes in single macromolecules or in cellular macromolecular structures such as membranes. Typical examples of physical memory devices are the magnetic tape of tape recorders. Katchalsky conceives of the possibility that conformational transitions in macromolecules might also exhibit hysteresis phenomena. Also, the existence of various distributions of functional side-groups along the macromolecules makes possible the existence of different domains with a wide range of transition points.[123] Metastable macromolecular states of high permanence are linked with two-dimensional hysteresis phenomena based on domains in biological films.

According to Katchalsky et al,[123] if we assume that the smallest number of nucleotides is about 8-10 in a domain,

the energy involved in a phase transition of a nucleotide is
higher than 2 RT, which is a plausible figure in case memory
recording in domain structures is considered. This figure
is sufficiently high to make a signal changing the domain
structure distinguishable from the thermal noise, but yet
small enough to be of use in the low-energy transformations
of living organisms. In this connection, it is pertinent
to point out that membranes as two-dimensional ordered arrays
of macromolecular assemblies, acting like information storage
devices, would ideally fulfill the dual criteria of "low
energy switching", and optimum size of domains as metastable
forms of high permanence.

Cooperativity of Membranes and their Subunit Structure.
Review of the data on membrane organization as revealed by
correlated ultrastructural and biochemical studies discloses
certain general features. Coherent paucimolecular layers of
indefinite lateral extension appear to consist of a periodic,
hydrated lipoprotein substrate which is integrated with
specific macromolecular repeating subunits organized in
asymmetric, "paracrystalline", arrays within the plane of the
layers. There is a fundamental underlying concept that
membranes are made up by the stereospecific association of
repeating macromolecular subunits, with specific conformations
dependent on their association with the membrane substrate.

Recently, J. P. Changeux and his colleagues[132] have extended
their theory of allosteric transitions in enzyme-substrate

reactions[125] to membranes composed of identical units,
applying some of the principles and approaches proposed for
regulatory enzymes. They discuss the cooperative properties
of membranes on the basis of their highly ordered structure.

Based primarily on data derived from correlated ultra-
structural and biochemical studies of membranes, carried
out during the past years by Fernández-Morán, Green, Perdue,
et al,[6,15,17] they consider the following significant aspects
of membrane organization: (a) membranes are made up by the
association of repeating macromolecular lipoprotein units;
(b) the conformation of these units (protomers) differs when
they are organized into a membrane structure or dispersed in
solution; (c) many biological or artificial lipoprotein
membranes respond in vivo, as well as in vitro, to the
binding of specific ligands by some modification of their
properties which reflects rearrangement of the membrane
organization and presumably of the repeating unit's
conformation.

From this they deduce a theoretical model of a biological
membrane as an ordered collection of repeating globular
lipoprotein units, or "protomers" organized into a two-
dimensional, crystalline lattice. The protomer constitutes
the "primitive cell" of the lattice and does not necessarily
possess in itself any particular symmetry properties.
Several conformational states are reversibly accessible to
the protomer. The conformation of the protomer depends upon

its association with neighboring protomers, and is thus
submitted to a lattice constraint similar to the quaternary
constraint involved in the organization of the quaternary
structure of oligomeric proteins. This interesting theory
has been primarily developed to account for the cooperative
phenomena accompanying the binding of ligands to a membrane.
This model predicts the two classes of responses exhibited
by biological membranes: a "graded" response or an "all-or-
none" response. Basic to their theory is the assumption
that "conformational changes of the protomer not only preexist
the ligand binding but are not fundamentally different whether
the ligand is bound or not." These authors emphasize that a
number of important biological phenomena seems to be related
to the highly cooperative structure of cell membranes.

Multiplex Systems and Possible Ultrastructure Correlates
in Nerve Membranes.

There appears to be a deep analogy between von Neumann's
multiplexing concept[124] (which essentially introduces re-
dundancy so that the reliability of the whole system is
greater than the reliability of its parts) and the hier-
archically organized redundancy of structural substrates
in the CNS at the macromolecular and molecular levels now
revealed by electron microscopy. The microcomponentry re-
quired by the multiplexing technique may partly correspond
to the immense number (in the order of ca. 10^{16} to 10^{19})
of specific macromolecular assemblies and related subunits
periodically arranged on the nerve membranes and derivatives

in close association with axon filaments, ribosomes, and
other key components.

Major Problems in the Study of Nerve Membrane Ultrastructure.

With the improved methodology now available it is tempting to
systematically extend the fine structure studies to all
regions of the nervous system in vertebrate and invertebrate
specimens.[126]   However, one should bear in mind that this
purely descriptive approach could easily consume the best
efforts of entire generations of investigators. Thus, even
if perfect techniques were currently available, a complete
morphological analysis of three-dimensional relationships
based on electron microscopy of serial sections would involve
such a prohibitively large number of images to map only a
few cubic centimeters of nerve tissues that total man hours
would figure in the order of centuries.

Instead, it appears more profitable to concentrate on
certain key problems of nerve ultrastructure and function:
(a) Correlated biochemical, electron microscopic and bio-
physical characterization of the major protein, lipoprotein,
glycoprotein components, and the macromolecular and multi-
enzyme assemblies associated with nerve membranes in a

variety of specimens, including human. Attention
would center on well-characterized enzymatic and protein
components with specific physiological properties.[127]

(b) Systematic studies of the organization of subunit patterns
within the plane of membrane layers, preferably examined in
the native hydrated state under conditions of reduced rad-
iation damage and of minimum perturbation, by using improved
instrumentation and preparation techniques for high resolu-
tion, low-temperature electron microscopy correlated with
polarized light and x-ray diffraction studies.[2,6,33,92,93,98,113,114,116]

(c) Correlation of ultrastructural studies with a comprehensive
"macromolecular and molecular anatomy program" aimed at
achieving reliable dissection and quantitative separation of
the entire spectrum of discrete molecular species using
Anderson's zonal centrifuge techniques[133] and related methods.

(d) Correlative studies of specific, dynamic changes on
selected nerve specimens in vivo[129] and in vitro[130](tissue
and organ cultures) using light and electron microscopy.

It would be of special interest to carry out a correlated
ultrastructural and biochemical analysis of the molecular
organization of selected key sites of the central nervous
system by performing a series of "submicroscopic or macro-
molecular biopsies". Such a submicroscopic biopsy, for
example of key synaptic sites or cerebral nuclei, can be

carried out "in vivo" using precision stereotactic techni-
ques under conditions of minimum perturbation without
inflicting more than "subliminal" damage, which is perhaps
hardly greater than the natural rate of decay of nerve
cells taking place in the adult brain. These submicroscopic
CNS biopsies, carried out with specially developed ultra-
microcapillaries, can be combined with subsequent tissue
culture explants and related in vitro techniques. Hydén[129]
has already carried out significant pioneering work in
microbiopsies of living tissues. Further development
of these techniques holds great potential for electron
microscopy.

Ultrastructural studies could also be combined with appro-
priate non-destructive techniques, including nuclear magne-
tic resonance,[34] electron spin resonance, and methods for
investigation of fast reactions.[100] Neutron diffraction
and scattering studies[97] suitably modified for examination
of nervous tissues should also yield valuable information.

Suggested Methodological Approaches in Correlated Studies of
Nerve Membranes.

Recent improvements in instrumentation and preparation tech-
niques for electron microscopy appear to be especially suited
for correlative investigations of nerve membrane ultrastructure
at the molecular and pauciatomic levels[116]: (a) High resolution
phase contrast electron microscopy with improved point cathode
sources, low temperature stages and specially stabilized power
supplies, as well as new types of phase contrast apertures
has enabled us to visualize directly for the first time (Fig. 13b)
the hexagonal cells of single crystal graphite containing only
10 carbon atoms. Demonstration of this capability of point-
to-point resolution in the range of interatomic distance now
makes it appear feasible to record detail of molecular and
pauciatomic dimensions in biological specimens. Extension
of this work and the enhanced capability of interpreting
phase contrast images should eventually enable us to achieve
direct readout of molecular and submolecular structures,
particularly in nerve membranes. Parallel advances in low-
temperature electron microscopy at liquid helium temperatures
using specially developed superconducting lenses,[113,114,116c]
image   intensifiers and improved recording techniques should
eventually permit us to see directly the three-dimensional
structure of key regions of synaptic junctions and of other
nerve membrane areas.

(b) By using vacuum-tight microchambers[92-98] with ultrathin windows and related techniques, it is possible to observe membranes under conditions approaching their native hydrated state. Examination of living hydrated specimens can now be carried out thanks to recent advances in high voltage electron microscopy.[115] Larger microchambers for examination of intact, viable biological specimens have been successfully applied owing to the far greater penetration now possible with advanced high voltage electron microscopes. Moreover, by using pulsed T-F emission or stroboscopic microbeam illumination, one can, in principle, obtain a unique combination of high spatial and high temporal resolution for the study of fast reactions in nerve membranes.

(c) Scanning electron microscopy can likewise be profitably used in the study of nerve membrane specimens examined under favorable conditions. As shown by the recent remarkable work of R. F. Pease et al,[131] electron micrographs of living specimens of various developmental stages of the insect Tribolium confusum have been obtained with a scanning electron microscope at magnifications of about 1,000 X. The requisite conditions of moderate vacuum and the relatively minor effects of irradiation by the imaging electron beam, which have been shown to be compatible with survival of certain specimens, may be applied in suitably designed experimental chambers to study selected nerve membranes and nerve cell specimens. Scanning electron

microscopy can also be extended to the application of special bioelectric and biomagnetic probes for the study of rapid and ultrarapid phenomena in nerve membrane systems.

(d) We can also foresee the unique potential of electron microscopy "applied in reverse" for achieving electron optical demagnification of letters, diagrams, printed circuits, etc., in such a way that in the optimal case, the contents of a several million volume library can be condensed on an area of a single page size or its equivalent.[117,118]

(e) The same electron optical techniques which revealed the exquisite molecular componentry of nerve cells may some day serve to duplicate true homologous ultraminiaturized, "molecular computer and information storage systems." Although the packing density embodied in the human brain is of the order of 10^9 components per cubic inch, impressive advances made during the past years in manufacturing integrated microelectronic systems have already resulted in practical packing densities of about 10^5 elements per cubic inch, which is susceptible of further improvement. Likewise, functional printed circuits of micron or submicron dimensions which could fit on a red blood cell, for example, and transmit important functional data recorded in vivo at the cellular and sub-cellular levels are within the scope of present technology.

One can conceive of integrated ultramicroelectric circuits which begin to approach the dimensions of macro-molecular assemblies, and could eventually be incorporated

into key junctional sites of living nerve membranes without undue perturbation. Such submicroscopic prosthetic sensors, with their envelopes composed of biosynthetically, produced macromolecular "protein coats" to form integral components of the nervous systems, could well subserve certain unique functions. For example, since they can be produced in large quantities and implanted in sufficient numbers throughout the central nervous system, they can provide a direct operational link at the crucial macromolecular level between the central nervous system and man-made information processing systems, such as computers of commensurate complexity.

There is increasing evidence that biological membrane systems may have properties in common with semiconductors.[2,119,120] It may therefore be of interest to reconsider as a working hypothesis suggestive of new experimental approaches our earlier speculations on certain specific crystalline properties such as piezoelectric effects, semiconductor properties or equivalent phenomena which may be associated with the fluid-crystalline nature of myelin and related lamellar systems.[2,30] In connection with correlated studies on basic mechanisms of electoencephalography, I had previously discussed the hypothetical possibility that myelin and other paracrystalline nerve membrane systems might function as generators or specific amplifiers of coherent electromagnetic radiation, mainly in the infrared or UHF range between 1 and about 10 microns wavelength.[134] Similar hypothetical mechanisms involving far infrared quanta and

coherent phenomena in the transfer of biological information on the macro- and supramolecular level are now being considered by J. Polonsky[135] in the scope of molecular exciton theory and quantum electronics.

Conclusions

Obviously, our current knowledge about nerve membrane ultra-structure and function is but a presage of many as yet unknown and unexpected properties of these basic constituents of the nervous system, awaiting further investigation.

Ultrastructure research should eventually enable us to learn how all of the prodigious activity of nerve cells and their appendages may be unfolding invisibly at the molecular and atomic levels in a strange, spatial-temporal framework, possessing an order that one may some day aspire to comprehend.

The old adage may thus prove to be invested with a deeper meaning when the simile is invoked that the neurons apparently have so little to show for all their incessant submicroscopic labors, because, like the mills of the gods, they grind exceedingly small--between membrane interfaces at the molecular level.

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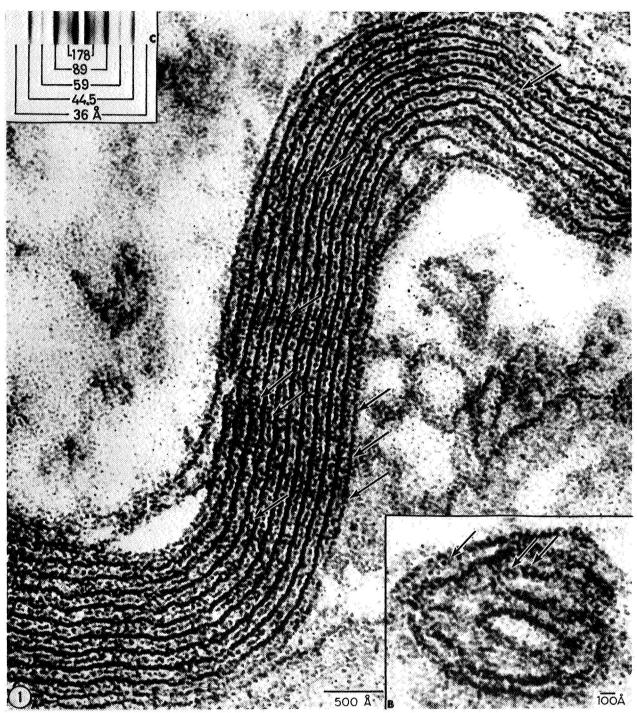
FIG.1 (a) High-resolution electron micrograph of myelin sheath segment from transverse thin section of frog sciatic nerve showing concentric array of dense and intermediate layers. Particulate subunit structures (arrows) are regularly found within the plane of the layers in these well-preserved, osmium fixed, low-temperature preparations. X 480,000. (b) Axonal mitochondria from a similar preparation demonstrating globular subunits in the dense layers of the cristae. X 600,000. (c) Low-angle x-ray diffraction pattern of fresh rat sciatic nerve featuring a fundamental period of 178Å, with typical alternation of the intensities of the even and odd orders.

FIG.2 (a) High-resolution micrograph of thin myelin sheath segment from transverse section of frog sciatic nerve, fixed and embedded at low temperatures. The characteristic particulate fine structure (arrows) detected mainly in the dense intermediate layers is also revealed by other techniques yielding improved preservation of the highly regular laminated sheath structure. X 900,000.
(b,c) Ultrathin frozen section of fresh frog sciatic nerve negatively stained with phosphotungstate and examined by low temperature electron microscopy. These repeating particulate units (arrows) attached to the dense myelin layers have consistently been found only in negatively stained, unfixed, specimens. Bearing in mind the inherent artifact possibilities, their possible relationship to the subunit structures, already shown by other methods, is now being further investigated. X 1,300,000.
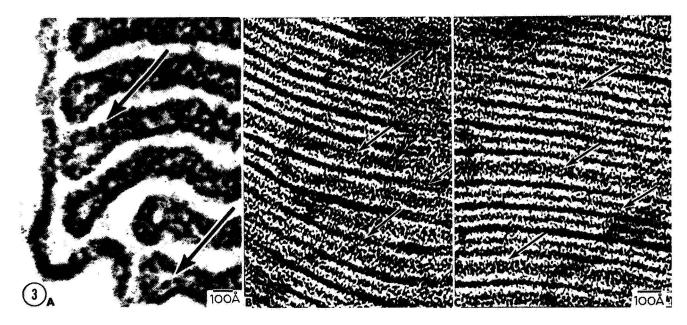
FIG. 3 High-resolution electron micrographs of thin sections of frog retinal rod outer segments:
(a) Osmium-fixed, low-temperature preparation of dark-adapted retina showing arrays of dense
particles about 40Å in diameter (arrows) within the compound membrane of the unit discs, X700,000;
(b,c) ultrathin sections of fresh, unfixed, light-adapted rod outer segments prepared without
embedding by improved techniques, and stained with uranyl formate. The ordered spherical particles
with indications of substructure of 10-15Å (arrows) can be detected in the dense and intermediate
membrane layers. The presence of an ordered substructure within these membranes is substantiated
by recent x-ray diffraction studies. X 900,000.
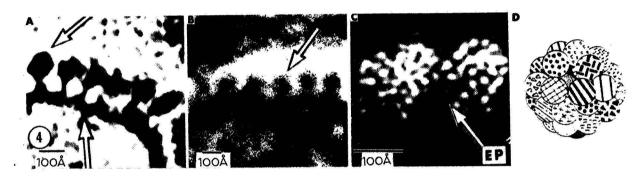
FIG. 4. Electron micrographs of: (a)enlarged segment of crista from negatively stained beef heart mitochondrion demonstrating three parts of the elementary particle (EP): head piece, stalk,and base piece, X 900,000; (b)segment of crista from thin mitochondrial section showing arrays of similar particles, X 700,000; (c)particulate units of approximately the same size as the EP,containing the electron transfer chain, isolated from beef heart mitochondria by Dr. D.E. Green et al., X 2,000,000. (d)Diagrammatic illustration of a macromolecular assembly containing many species of macromolecular components. Courtesy of Professor F.O.Schmitt.
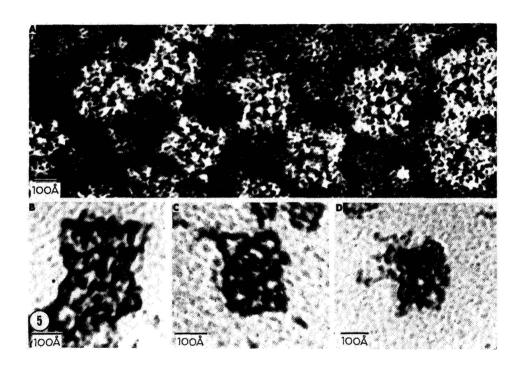
FIG.5. High resolution electron micrographs of: (a) E. coli pyruvate dehydrogenase complex (PDC) prepared by Dr. L.J. Reed et al. showing characteristic central tetrad and associated subunits of this multienzyme complex negatively stained with phosphotungstate, and (b) positively stained with uranyl acetate, X 1,300,000. (c,d) E. coli α-ketoglutarate dehydrogenase complex (KGDC) isolated in Dr. Reed's laboratory displaying similar structural features of the positively stained particles in different orientations, X 1,400,000.
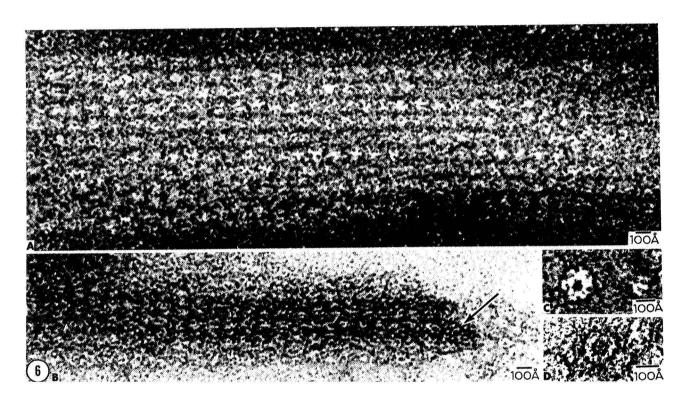
FIG. 6. Electron micrographs of bovine L-glutamate dehydrogenase (GDH)(Boehringer & Soehne,Mannheim): (a)microcrystals negatively stained with uranyl formate by microdroplet cross-spraying technique showing typical periodic lattice formed through ordered aggregation of the constituent molecules, X 600,000; (b)in thinner, ribbon-like strands the regular substructure of individual molecules (arrow) can be discerned, X 600,000; (c,d) as a result of the improved preservation and enhanced resolution achieved in these preparations, undissociated individual molecules(ca. 1 million molecular weight) are frequently detected, appearing as spherical or polyhedral particles of diameter 130 - 150 Å in different orientations. Subunits of triangular shape and size are also found. X 900,000. In addition to its specific value investigation of this enzyme is also of interest as a model system to illustrate the different states of molecular and supramolecular association bearing on the general organization of enzyme complexes in membranes
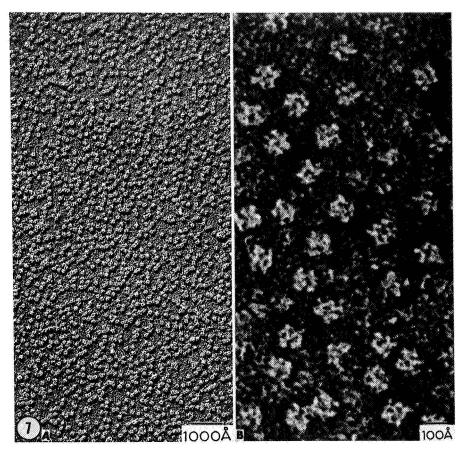
FIG. 7. (a)Electron micrograph of shadow-cast particles of Fraction I protein isolated from Chinese cabbage leaves by F.J. Kieras and R. Haselkorn. This major soluble protein fraction located predominantly in the chloroplasts has carboxydismutase activity. As seen in negatively stained preparations (b) the individual particle appears to be a cube, about 120Å along each edge, containing 24 subunits. This is one of the first demonstrations that the substructure of a protein enzyme can be directly resolved in the size range of the enzyme complexes associated with membrane systems.
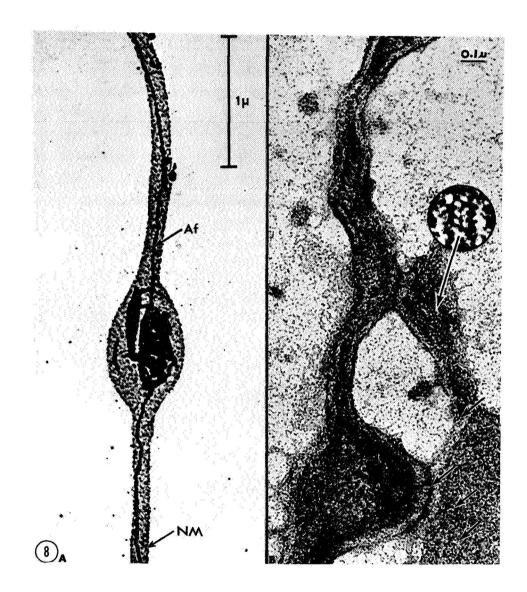
FIG. 8. Electron micrographs of: (a) fine submicroscopic nerve fiber from the lateral funiculus of the frog spinal cord. The fiber shaft has a width of 1000 Å and consists of a flattened, ultrathin tubular membrane (NM) enclosing an individual axon filament (Af) 200 Å in diameter. X 54,000; (b)Submicroscopic nerve fiber ending and associated membrane components isolated from fresh frog spinal cord,stained with uranyl formate, and examined by low-temperature electron microscopy. These well-preserved ultrathin nerve membrane preparations exhibit a characteristic surface structure featuring in certain regions hexagonal arrays of closely packed polygonal elements, 60 to 90 Å in diameter (insert).Various types of larger particulate components (arrows) are found attached to the membranes, particularly in junctional regions. X 100,000.   Insert: X 600,000.
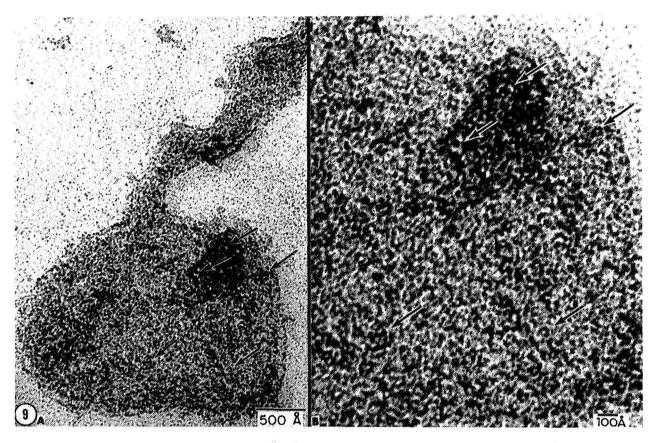
FIG. 9. High resolution electron micrographs of: (a) submicroscopic nerve fiber ending isolated from fresh frog spinal cord using improved preparation techniques which permit examination of whole mounts of ultrathin nerve membranes, without introducing the fixation, extraction, and embedding artifacts inherent in standard thin-sectioning methods. Under these favorable conditions an underlying subunit structure of the membrane surfaces can be resolved, with associated electron-dense components (arrows),X 400,000; (b) as shown in the enlarged segment of this membrane, numerous polygonal components with electron-dense or annular cores (arrows), 100 to 200 Å in diameter, contribute to the mosaic-like surface pattern, X 800,000. Further methodological improvements should eventually make it possible to directly observe the molecular organization of individual nerve membranes in their native hydrated state.
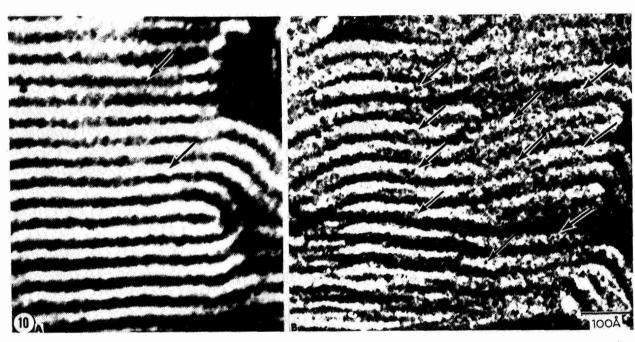
FIG. 10. Low-temperature electron micrographs of: (a) lecithin micelles embedded in thin, buffered phosphotungstate film showing periodic dense lines separated by light bands corresponding to the hydrophilic and hydrophobic regions of adjacent bimolecular leaflets; (b) lecithin micelles similarly embedded in thin phosphotungstate film, and exposed to argon gas under pressure, showing dense particulate aggregates (arrows) along the dense and intermediate layers, which may represent the resulting argon hydrate microcrystals in the size range of 10 -20 Å. X 2,000,000. Model experiments of this kind could provide direct experimental evidence for the existence of hydrate microcrystals forming reversibly in nerve membranes as first postulated by L. Pauling.

FIG. 11. Electron micrograph of thin section through retinulae from a tropical "Skipper" butterfly demonstrating the highly regular organization of the differentiated membrane-bound components in these photoreceptors. The matched rhabdomere pairs (arrows-R) are surrounded by rows of mitochondria(Mi),grouped together in radial columns by a network of fine tubular channels(Ut) arising from the tracheal compartments (T).The walls of the tubular rhabdomere compartments (insert) contain arrays of dense particles which may correspond to the ordered arrangement of the dichroic photopigment molecules providing the basis for the polarization-analyzing property of the insect eye.
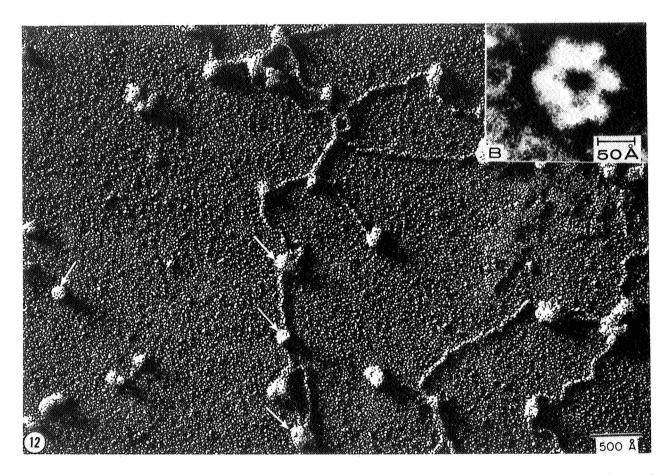
FIG. 12. Electron micrographs showing: (a) characteristic attachment of RNA polymerase molecules (arrows) to DNA strands as seen in shadowed preparations, X 400,000 ; (b) DNA-dependent RNA polymerase molecules from E. coli prepared by the Chamberlin & Berg procedure appear to consist in negatively stained preparations of six subunits arranged in a hexagon with a cross-section of 120 to 130 Å. These correlated electron microscopic and biochemical studies are expected to yield further information on RNA polymerase and its participation in the differential RNA transcription upon DNA templates,which is of fundamental importance in the regulation of protein synthesis and function in nerve cell membranes and their derivatives.
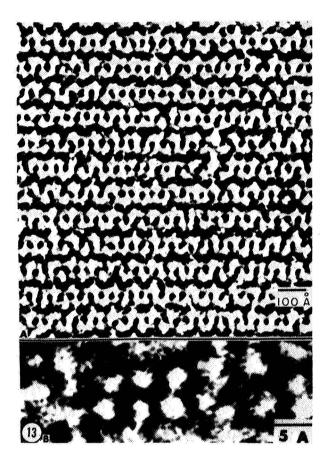
FIG. 13. High resolution electron micrographs showing: (a) the complex arrangement of the structural subunits of about 8 - 20 Å within the periodic lattice of catalase crystals stained with uranyl formate, X 1,200,000 ; (b) phase contrast image of the hexagonal array of carbon atoms in ultrathin layer of single-crystal graphite, X 32,000,000. This level of resolution of pauciatomic structural patterns, which can now be achieved under certain conditions with improved instrumentation and preparation techniques, should prove to be of key value in correlated studies for direct visualization of the molecular organization of nerve membranes and associated enzyme systems.

FIGURE CAPTIONS

Fig. 1.   (a) High resolution electron micrograph of myelin
          sheath segment from transverse thin section of frog
          sciatic nerve showing concentric array of dense and
          intermediate layers. Particulate sub-unit structures
          (arrows) are regularly found within the plane of the
          layers in these well-preserved, osmium fixed, low-
          temperature preparations. X 320,000. (b) Axonal
          mitochondria from a similar preparation demonstrating
          globular subunits in the dense layers of the cristae.
          X 400,000. (c) Low-angle x-ray diffraction pattern
          of fresh rat sciatic nerve featuring a fundamental
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Fig. 2.   (a) High resolution micrograph of thin myelin
          sheath segment from transverse section of frog
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particulate units (arrows) attached to the dense

myelin layers have consistently been found only in

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Fig. 3. High resolution electron micrographs of thin sections

of frog retinal rod outer segments: (a) Osmium-fixed,

low-temperature preparation of dark-adapted retina

showing arrays of dense particles about 40 $\overset{\circ}{A}$ in

diameter (arrows) within the compound membrane of

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of fresh, unfixed, light-adapted rod outer segments

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Insert: X 354,000.

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attachment of RNA polymerase molecules (arrows) to
DNA strands as seen in shadowed preparations, X 284,000;
(b) DNA-dependent RNA polymerase molecules from E. coli
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MEMBRANE ULTRASTRUCTURE IN NERVE CELLS - H. Fernández-Morán

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